Mechanisms of Signal Transduction:

Cross-talk between JIP3 and JIP1 during Glucose Deprivation: SEK1-JNK2 AND Akt1 ACT AS MEDIATORS

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Cross-talk between JIP3 and JIP1 during Glucose Deprivation

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We have previously observed that glucose deprivation activates the ASK1-MEK-MAPK signal transduction pathway. In the present study, we reveal that two scaffolding proteins, JIP1 and JIP3, have a cross-talk that leads to the regulation of the ASK1-SEK1-JNK signal during glucose deprivation. Glucose deprivation rapidly increases the interaction between ASK1 and JIP3, and the consequently activated ASK1 phosphorylates SEK1 on the Thr-261 residue. The activated SEK1 dissociates from JIP3 and phosphorylates JNK2 on the Tyr-185 residue. Phosphorylated JNK2 binds to JIP1, and the phosphorylation of the Thr-183 residue of JNK2 occurs. JNK2 phosphorylates JIP1 on the Thr-103 residue and leads to dissociation of Akt1 from JIP1. Dissociated Akt1 binds to SEK1 and ASK1 and inhibits their enzyme activity by phosphorylating SEK1 on the Ser-80 residue and ASK1 on the Ser-83 residue. Taken together, our data demonstrate that cross-talk between JIP3 and JIP1 is mediated through SEK1-JNK2 and Akt1.

Cellular responses to external signals should accurately proceed without errors. Mitogen-activated protein kinases (MAPKs)[1] are components of pathways that relay signals mediating proliferation, survival, death, or cell cycle arrest to particular cell compartments in response to a diverse array of extracellular stimuli (1). Their ubiquity and versatility raise the issue of how they achieve specific coupling of signal to cellular response (2). Most prominent among the known signal transduction pathways that control these events are the MAPK cascades, whose components are evolutionarily highly conserved in structure and organization, each cascade consisting of a module of three cytoplasmic kinases: a MAPK kinase kinase, a MAPK kinase, and the MAPK itself (3). The control of diverse cellular processes in response to a plethora of extracellular stimuli by a few MAPKs lies in the fact that considerable specificity is built into MAPK activation and function (4). There are two possible mechanisms that can operate in the cell. First, the protein kinase forming the MAPK signaling modules may interact via a series of sequential binary interactions to create a protein kinase cascade, or scaffold proteins may mediate signaling cascades by organizing the components of MAPK signaling into modules (5). Scaffold proteins have been proposed to serve as organizing centers for signal transduction, and one function of scaffold proteins might be to reduce the extent of cross-talk between different pathways sharing molecular components (6).

We have previously observed that glucose deprivation activates the ASK1-MEK-MAPK signal transduction pathway. In this study, we hypothesized that the ASK1-MEK-MAPK signal transduction can be modulated through cross-talk between two scaffold proteins during prolonged glucose deprivation, as represented schematically in Fig. 12. The first scaffold protein, JIP3 (JNK-interacting protein 3, also named JSAP1 (JNK/SAPK-associated protein 1)), a c-Jun N-terminal kinase (JNK)-binding protein (7), can be phosphorylated by ASK1, and its phosphorylation facilitates the recruitment of stress-activated protein kinase/extracellular-signal regulated kinase (SEK1), MKK7, and JNK into the JIP3-ASK1 signaling complex (8). The binding between JIP3 and JNKs (including JNK1, -2, and -3) is specific, whereas there is either no binding or very low binding of ERK2 and p38α to JIP3 (7). The second scaffold protein, JIP1, is a scaffold protein that integrates both positive and negative regulators of JNK. JIP1 assembles JNK, MKK7, and mixed lineage protein kinase proteins on different regions of JIP1 and facilitates the JNK signaling pathway (9, 10). Recent studies have revealed that recruitment of JNK to JIP1 and phosphorylation of JIP1 by JNK are prerequisites for activation of the JNK module (11). Moreover, JNK activity can be antagonized by Akt kinase activity in numerous cellular systems (12–15). In the present study, we observed that JNK activation was regulated by cross-talk between JIP3-associated proteins and JIP1-associated proteins during prolonged glucose deprivation. JIP3-mediated activation of SEK1 and JIP1-mediated restoration of Akt1 activity play an important role in the regulation of the ASK1-MEK-MAPK signal transduction pathway during prolonged glucose deprivation.

MATERIALS AND METHODS

Cell Culture—Human prostate adenocarcinoma DU-145 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT) and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a humidified atmosphere containing 5% CO_2 and air at 37 °C.

Reagents and Antibodies—Polyclonal anti-SEK1, anti-phospho-Thr-261-SEK1, anti-phospho-Ser-80-SEK1, anti-phospho-Ser-83-ASK1, anti-phospho-Thr-308-Akt, anti-phospho-Ser-473-Akt, anti-Akt, anti-Bad, and anti-phospho-Ser-136-Bad were purchased from Cell Signaling (Beverly, MA), and anti-ACTIVE (phospho-Thr-183 and phospho-Tyr-

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[S] The on-line version of this article (available at http://www.jbc.org) contains an additional figure.

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase kinase; siRNA, small interfering RNA; MOI, multiplicity of infection; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; DTT, dithiothreitol; DLK, dual zipper-bearing kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; GST, glutathione *S*-transferase.

185) JNK was purchased from Promega (Madison, WI). Monoclonal antibodies were purchased from the following companies: anti-JIP3 and anti-JIP1 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-actin from ICN (Costa Mesa, CA), anti-HA (clone 3F10) from Roche Applied Science, anti-FLAG (clone M2; mouse) from Sigma, and anti-His (penta-His; mouse) from Qiagen (Valencia, CA). Other chemicals were purchased from Sigma.

Shuttle Vector Construction—pcDNA3-HA-ASK1 was kindly provided by Dr. Ichijo (Tokyo Medical and Dental University, Tokyo, Japan). pAdlox-HA-ASK1 was made by inserting the SpeI/XbaI fragment from pcDNA3-HA-ASK1 into XbaI-cut pAdlox. pcDNA3-Myc-ASK1 was made by inserting the PCR product of ASK1 to pcDNA3-Myc. Sense primer was 5′-ATTATACGTATAGCACGGAG-GCGGACGAGGG-3′, introducing an SnaBI site, and antisense primer was 5′-CGCGTCTAG ATCAAGTCTGTTTGTTTCGAAAGTCAATG-3′, introducing an XbaI site for inserting into pcDNA3-Myc (BamHI → Klenow → XbaI). pAdlox-Myc-ASK1 was made by inserting the SpeI/XbaI fragment from pcDNA3-Myc-ASK1 into SpeI/XbaI-cut pAdlox. pcDNA3.1-His C-SEK1 was made by inserting a BamHI fragment from pEBG-SEK1. Adlox-His-SEK1 was made by inserting an SpeI/XbaI fragment from pcDNA3.1-His C-SEK1 into SpeI/XbaI-cut pAdlox shuttle vector. pFLAG-CMV5 MKK7α1, -γ1, and -γ2 was kindly provided by Dr. Hiroshi Nishina (University of Tokyo). After digestion with EcoRI/SmaI, their fragments were inserted into the EcoRI/EcoRV site of pcDNA3.1His C. pAdlox-His-MKK7α1, -γ1, and -γ2 were made by inserting the HindIII/XbaI-digested His-MKK7α1, -γ1, and -γ2 fragments into pAdlox. pLNCX-3X HA-p46 JNK1 α and pLNCX-HA p54 JNK2 α were kindly provided by Lynn E. Heasley (University of Colorado Health Sciences Center, Denver, CO). pLNCX-3X HA-p46 JNK1 α and pLNCX-HA p54 JNK2 α were digested with HindIII/ClaI, and their fragments were subcloned into HindIII/AccI-digested pAdlox. pAdlox-His-JNK1 was also made by inserting an SpeI/XbaI fragment from pcDNA3.1-His A-JNK1 into SpeI/XbaI-cut pAdlox. pCMV5-FLAG-JIP1 was kindly provided by Dr. R. Davis (University of Massachusetts Medical School, Worcester, MA). pFLAG-CMV2-JIP1, which was FLAG-tagged at its N-terminal and restriction enzyme recognition sites at the flanking sides (5′, HindIII; 3′, XbaI) was produced by PCR using the pCMV5-FLAG-JIP1 as template. Sense primer was 5′- TAATA-AGCTTGCGGAGCGAGAGAGCGGCCTG-3′, and antisense primer was 5′-GCCGTCTAGACTACTCCAAGTAGATATCTTC-3′. pAdlox-FLAG-JIP1 was produced by inserting the SpeI/XbaI fragment from FLAG-CMV2JIP1 into SpeI/XbaI-cut pAdlox shuttle vector. pcDNA3-FLAG-JIP3 was kindly provided by Dr. Yoshioka (Kanazawa University, Japan). pAdlox-FLAG-JIP3, which was FLAG-tagged at its N-terminal and restriction enzyme recognition sites at the flanking sides (5′, SphI; 3′, AccI) was produced by PCR using the pcDNA3-FLAG-JIP3 as template. Sense primer was 5′-CTGCGCAT-GCTGATGGACTACAAAGACGATGACGACAAGCT-3′, and antisense primer was 5′-CATCTAGTCGACTCACTCAGGGGTGTAGGACAC-CTGCC-3′. Adlox-HA-Akt1 (wild type Akt1 or kinase-inactive Akt1 (K179M)) was produced by inserting an HindIII/EcoRI fragment from pCMV6-HA-Akt1 into HindIII/EcoRI-cut pAdlox shuttle vector.

Adenoviral Vector Construction—All recombinant adenoviruses were constructed by employing the *Cre-lox* recombination system (16). The selective cell line CRE8 has an β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. Transfections were done by using Lipofectamine reagent (Invitrogen). 5×10^5 cells were split into a 6-well plate 1 day before transfection. For the production of recombinant adenovirus, 2 μg of SfiI-digested Adlox/FLAG-JIP1, Adlox/FLAG-JIP3, Adlox/His-SEK1, Adlox/His-MKK7α1, Adlox/His-MKK7γ1, Adlox/His-MKK7γ2, Adlox/HA-JNK1, Adlox/HA-JNK2, or Adlox/HA-Akt1, ψ5 viral genomic DNA (2 μg) were co-transfected into CRE8 cells. The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and ψ5 viral DNA. The new virus had an intact packaging site and carried a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of various types of shuttle vector to adenovirus was confirmed by Western blot analysis, after infection of corresponding recombinant adenovirus into DU-145 cells.

Site-directed Mutagenesis—The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to make point mutations in SEK1 protein. Threonine residue in mouse SEK1 was replaced with alanine (T259A). Sense primer oligonucleotide (5′-GACTCTATT-GCCAAGGCAAGAGATGCTGGGTGT-3′) and antisense primer oligonucleotide (5′-ACACCCAGCATCTCTTGCCTTGGCAATAGAGTC-3′) were used for site-directed mutagenesis. PCR was prepared by adding 5 μl of 10× reaction buffer, 20 ng of double-stranded DNA template

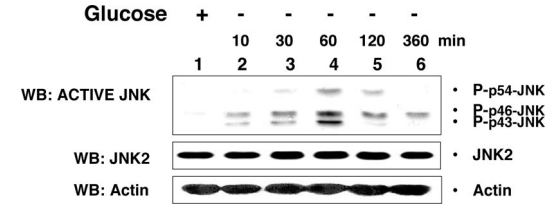

FIG. 1. **Activation of JNK during glucose deprivation.** DU-145 cells were exposed to glucose-free medium for various times (10–360 min). After incubation, cells were harvested, and Western blot analysis (*WB*) was performed with anti-ACTIVE JNK, anti-JNK2, or anti-actin antibody as the loading control.

(pAdlox-His-SEK1), 125 ng of each sense primer, 125 ng of each antisense primer, 1 μl of dNTP mix, double-distilled water to a final volume of 50 μl, and 1 μl of *Pfu* Turbo DNA polymerase (2.5 units/μl). PCR was performed with 14 cycles (95 °C for 30 s, 58 °C for 1 min, 68 °C for 7 min) with initial incubation at 95 °C for 30 s. Following temperature cycling, the reaction was placed on ice for 2 min to cool the reaction. After PCR, 1 μl of DpnI restriction enzyme (10 units/μl) was added directly to each amplification reaction and incubated at 37 °C for 1 h to digest the parental supercoiled double-stranded DNA. The DpnI-treated double-stranded DNA was transformed into *Epicurian Coli* XL1-Blue supercompetent cells. Colonies were selected, and each plasmid (pAdlox-HA-JNK2) was sequenced using primer (5′-GGATGCTA-ACTTATGTCAGG-3′) to confirm mutation.

RNA Interference by siRNA of SEK1, JIP1, or JIP3—To stably express siRNA for the long term knockdown, p*Silencer* 2.1-U6 hygro vector (Ambion, Inc., Austin, TX) was used for clonal cell lines. The inserts for hairpin siRNA into p*Silencer* were prepared by annealing two oligonucleotides. For human SEK1 siRNA, the top strand sequence was 5′-GATCCACGCAAAGCACTGAAGTTGTTCAAGAGACAACTTC-AGTGCTTTGCGTTTTTTTGGAAA-3′, and the bottom strand sequence was 5′-AGCTTTTCCAAAAAAACGCAAAGCACTGAAGTTGTCTCTT-GAACAACTTCAGTGCTTTGCGTG-3′. For human JIP1 siRNA, the top strand sequence was 5′-GATCCGACCTCTCGGAGATCACTGTT-CAAGAGACAGTGATCTCCGAGAGGTCTTTTTTGGAAA-3′, and the bottom strand sequence was 5′-AGCTTTTCCAAAAAAGACCTCTCGG-AGATCACTGTCTCTTGAACAGTGATCTCCGAGAGGTCG-3′. The annealed insert was cloned into p*Silencer* 2.1-U6 hygro digested with BamHI and HindIII. The correct structure of p*Silencer* 2.1-U6 hygro-SEK1 or *pSilencer* 2.1-U6 hygro-JIP1 was confirmed by nucleotide sequencing. The resultant plasmid, p*Silencer*-SEK1 or p*Silencer*-JIP1, was transfected into DU-145 cells, and hygromycin B (250 μg/ml)-resistant cell clones were isolated. The interference of SEK1 or JIP1 protein expression was confirmed by immunoblot using anti-SEK1 or anti-JIP1 antibody.

To down-regulate the JIP3, JIP3 siRNA (Santa Cruz Biotechnology) was used. Cells were transfected with JIP3 siRNA and incubated for 36 h. The interference of JIP3 protein expression was confirmed by immunoblot using anti-JIP3 antibody (Upstate, Charlottesville, VA).

Interaction between Proteins—To examine the interaction between ASK1 and JIP3/JIP1, adenoviruses expressing HA-tagged ASK1 (Ad.HA-ASK1) and FLAG-tagged JIP3/JIP1 (Ad.FLAG-JIP3 or JIP1) at an MOI of 10 were infected into DU-145 cells in 10-cm culture plates, respectively. For immunoprecipitation, after 48 h of infection, cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 80 μM aprotinin, 2 mM leupeptin, and the lysates were incubated with 1 μg of anti-HA (clone 3F10; Roche Applied Science) or 3 μg of anti-FLAG M2 mouse IgG1 (Sigma) for 2 h. After the addition of protein G-agarose (Santa Cruz Biotechnology), the lysates were incubated for an additional 2 h. The beads were washed three times with the lysis buffer, separated by SDS-PAGE, and immunoblotted with mouse anti-FLAG or mouse anti-HA (clone 12CA5; Roche Applied Science). Proteins in the membranes were then visualized using the enhanced chemiluminescence (ECL) reagent as recommended by the manufacturer (Amersham Biosciences).

To examine the interaction between SEK1/MKK7α1/JNK1 and JIP1/JIP3, anti-His antibody was used for immunoprecipitation. To examine the interaction between JNK2 and JIP1/JIP3, anti-HA antibody was used for immunoprecipitation.

Immune Complex Kinase Assay—For *in vitro* kinase assay of Akt1, DU-145 cells were lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and

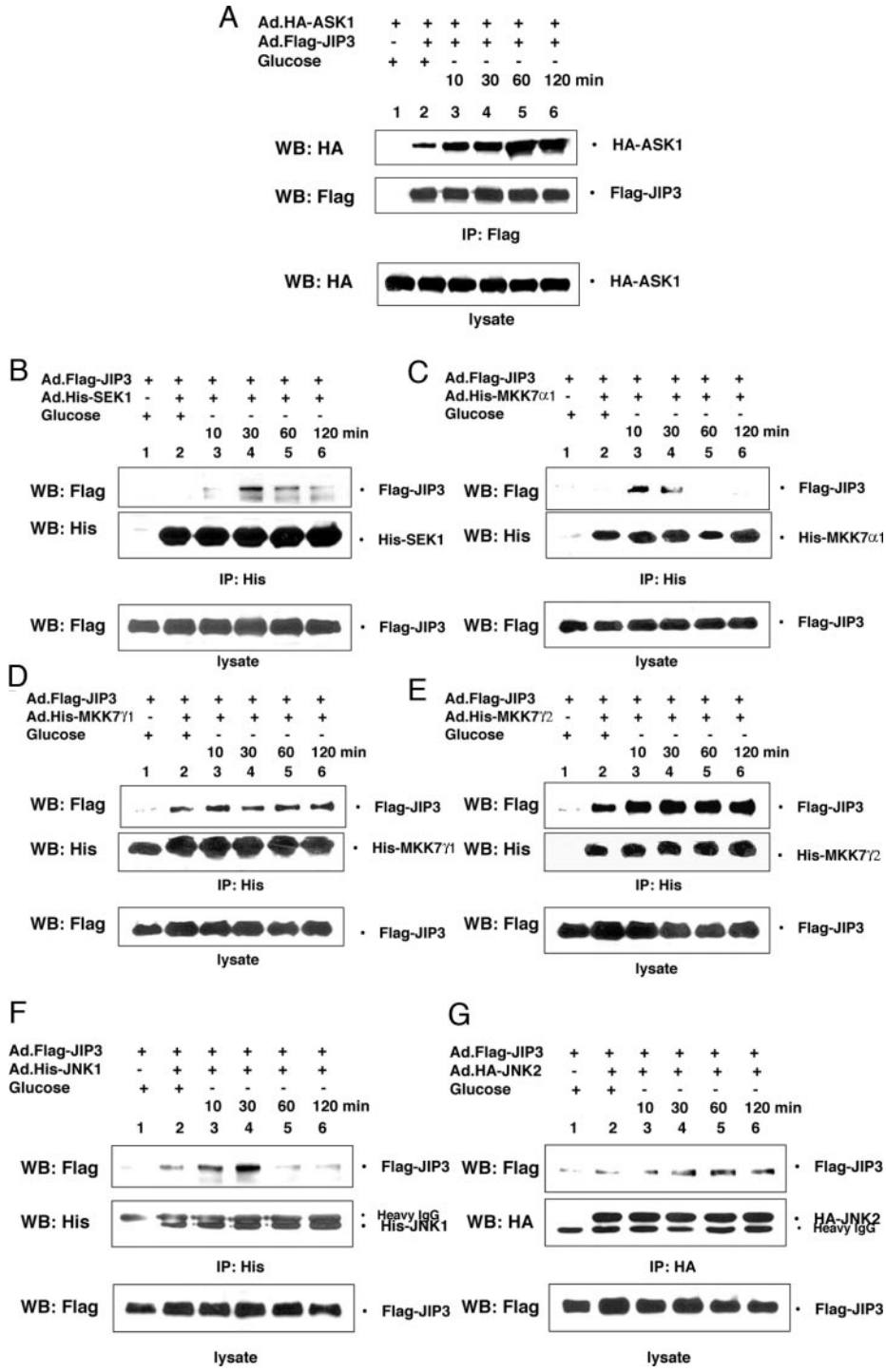

FIG. 2. **Interaction between JIP3 and various types of the ASK1-MEK-JNK signaling proteins during glucose deprivation.** DU-145 cells were co-infected with adenoviral vector containing FLAG-tagged JIP3 and HA-ASK1, His-MKK7α1, His-MKK7γ1, His-MKK7γ2, His-SEK1, HA-JNK1, or HA-JNK2 at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for various times (10–120 min). Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody (*A*), anti-His antibody (*B–F*), or anti-HA antibody (*G*) and immunoblotted (*WB*) with anti-FLAG, anti-His, or anti-HA antibody (*upper panels*). The presence of HA-ASK1 or FLAG-JIP3 in the lysates was verified by immunoblotting (*lower panels*).

protein inhibitor mixture solution (Sigma). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with mouse Akt antibody (Cell Signaling) and protein G-agarose (Santa Cruz Biotechnology). The beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution and washed once with the kinase buffer solution, and then they were subjected to kinase assays. To examine the Akt1 catalytic activity, GST-tagged fusion Bad protein (Santa Cruz Biotechnology) was used as a substrate of Akt1. 1 μg of Bad was incubated with immunoprecipitated Akt1 in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 2 mM DTT, and 20 μM ATP at 30 °C for 1 h. Finally, the reaction was stopped by adding 2× Laemmli buffer. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by immunoblotting using anti-phospho-Bad (Ser-136) antibody (Cell Signaling Technology, Inc., Beverly, MA). For *in vitro* kinase assay of SEK1/ASK1 phosphorylation by Akt1, DU-145 cells were co-infected with Ad.His-SEK1/Ad.Myc-ASK1 and Ad.HA-Akt1 at an MOI of 10. After 48 h of infection, cells were incubated in glucose-free medium for 1 h and then lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution (Sigma). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with anti-His/anti-Myc antibody and protein G-agarose. The beads were washed three times with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF and protein inhibitor mixture solution and washed once with the kinase buffer solution, and then they were subjected to kinase assays. 0.5 μg of GST-JNK/GST-SEK1 was incubated with immunoprecipitated His-SEK1/Myc-ASK1 in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 2 mM DTT,

TABLE I
Summary of data from experiments shown in Figs. 2 and 3

The relative ratio of the intensity of each kinase is plotted as a function of various periods of incubation in glucose-free medium. The data are a compilation of two or three separate experiments.

	ASK1	SEK1	MKK7α1	MKK7γ1	MKK7γ2	JNK1	JNK2
JIP3							
0 min	+	−	−	+	+	+	+
10 min	++	+	++	+	++	++	+
30 min	++	+++	++	+	++	+++	+
60 min	++++	++	−	+	++	−	+
120 min	+++	+	−	+	++	−	+
JIP1							
0 min	−	−	−	+	+	++	+
10 min	−	−	−	+	+++	++	+
30 min	+	−	+	++	+++	++	+++
60 min	++	−	++	−	+++	++	+++
120 min	++	−	++	−	+++	++	+++

and 20 μM ATP at 30 °C for 1 h. Finally, the reaction was stopped by adding 2× Laemmli buffer. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by immunoblotting using anti-ACTIVE JNK antibody (Promega, Madison, WI) or anti-phospho-SEK1 (Thr-261) antibody (Cell Signaling Technology). For the *in vitro* kinase assay of JIP1 phosphorylation by JNK2, DU-145 cells were infected with various types of Ad.HA-JNK2 (wild type, T183A, and Y185F) at an MOI of 10. After 48 h of infection, cells were incubated in glucose-free medium for 1 h and then lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution (Sigma). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with rat anti-HA antibody and protein G-agarose. The beads were washed three times with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor mixture solution and washed once with the kinase buffer solution, and then they were subjected to kinase assays. 0.5 μg of GST-JIP1 or GST-JIP1 (T103A) was incubated with immunoprecipitated HA-JNK2 (WT, T183A, γ185F) in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 2 mM DTT, 20 μM ATP, and 100 μCi/ml [γ-^{32}P]ATP at 30 °C for 1 h. Finally, the reaction was stopped by adding 2× Laemmli buffer. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography.

Immunoblot Analysis—Cell lysates were subjected to electrophoresis on 10% polyacrylamide gels containing SDS under reducing conditions, and the proteins in the gels were transferred onto a polyvinylidine difluoride membrane. The membranes were incubated with 7% (w/v) skim milk in PBST (PBS containing 0.1% (v/v) Tween 20) and then reacted with primary antibodies. After washing three times with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG. Then the proteins were detected with the ECL reagent.

RESULTS

JNK Activation during Glucose Deprivation—We previously reported that glucose deprivation activates JNK. To examine the time course of JNK activation during glucose deprivation, DU-145 cells were exposed to glucose-free medium for various times (10–120 min). Fig. 1 shows that three isoforms of JNK (p43, p46, and p54) were phosphorylated during glucose deprivation. Phosphorylation of JNK occurred within 10–30 min, gradually increased until 60 min, and then decreased during glucose deprivation. We postulated that bistable systems in the cells are involved in the regulation of JNK activation during glucose deprivation. Bistability can result from cross-talking between scaffolding proteins. This possibility was further examined.

Interaction between JIP3/JIP1 and ASK1-MEK-JNK Signaling Proteins during Glucose Deprivation—Previous studies have shown that JIP3 interacts with ASK1 (8). In this study, we investigated whether glucose deprivation promotes the interaction between JIP3 and ASK1. Fig. 2*A* shows that ASK1 associated with JIP3 in the presence of glucose. The interaction between these proteins gradually increased during glucose deprivation and reached a maximum at 1 h of glucose deprivation. This and the following experiments are compared diagrammatically in Table I. We further examined the interaction between JIP3 and downstream molecules (SEK1, MKK7α1, MKK7γ1, MKK7γ2, JNK1, and JNK2) of ASK1 signaling (Fig. 2, *B–G*). Interestingly, the kinetics of interaction between them during glucose deprivation were not identical. SEK1, MKK7α1, and JNK1 rapidly interacted with JIP3, reached a maximum at up to 30 min, and then dissociated from JIP3 during prolonged glucose deprivation (Fig. 2, *B*, *C*, and *F*). In contrast, little or no change in the interaction between JIP3 and MKK7γ1/JNK2 was observed in the presence or absence of glucose (Fig. 2, *D* and *G*). On the contrary, interaction between MKK7γ2 and JIP3 during glucose deprivation increased and reached a maximum within 10 min and then the interaction was sustained. We further investigated the interaction between the ASK1-MEK-JNK signaling proteins and JIP1, another scaffolding protein (Fig. 3). Interaction between JIP1 and ASK1/MKK7α1 during glucose deprivation increased and reached a maximum at 60 min and then the interaction was sustained. Interaction between MKK7γ2 and JIP1 during glucose deprivation rapidly increased and reached a maximum at 60 min and then the interaction was sustained. Interaction between JIP1 and MKK7γ1/JNK2 increased, reached a maximum within 30–60 min, and then decreased during glucose deprivation (Fig. 3, *D* and *G*). On the contrary, interaction between JIP1 and SEK1 or JNK1 was not detectable or not changed, respectively (Figs. 3, *B* and *F*). We summarized our observations in Table I. In this table, we have noticed that SEK1 and JNK2 were distinctively different from other proteins in terms of interacting with scaffolding proteins during glucose deprivation. Thus, we postulated that these proteins act as mediators for cross-talking between scaffolding proteins.

Role of JIP3 in the Activation of SEK1 and JNK during Glucose Deprivation—Data from Fig. 2 show the interaction between JIP3 and downstream molecules of ASK1 signaling. We hypothesized that this interaction is essential for activating downstream molecules of ASK1 signaling during glucose deprivation. To test this hypothesis, DU-145 cells were transfected with JIP3 siRNA or mock siRNA. Fig. 4 shows that the expression of JIP3 was effectively inhibited by siJIP3. Glucose deprivation-induced SEK1 and JNK activation were also inhibited in siJIP3-transfected cells. These results reveal that JIP3 plays an important role in activation of SEK1 and JNK.

Role of ASK1 in the Interaction between JIP3 and SEK1 during Glucose Deprivation—Previous studies have shown that ASK1 phosphorylates JIP3 and its phosphorylation facilitates interactions of JIP3 with SEK1 (8). To examine whether ASK1 has an effect on the interaction between JIP3 and SEK1 during glucose deprivation, cells were infected with adenoviral vector

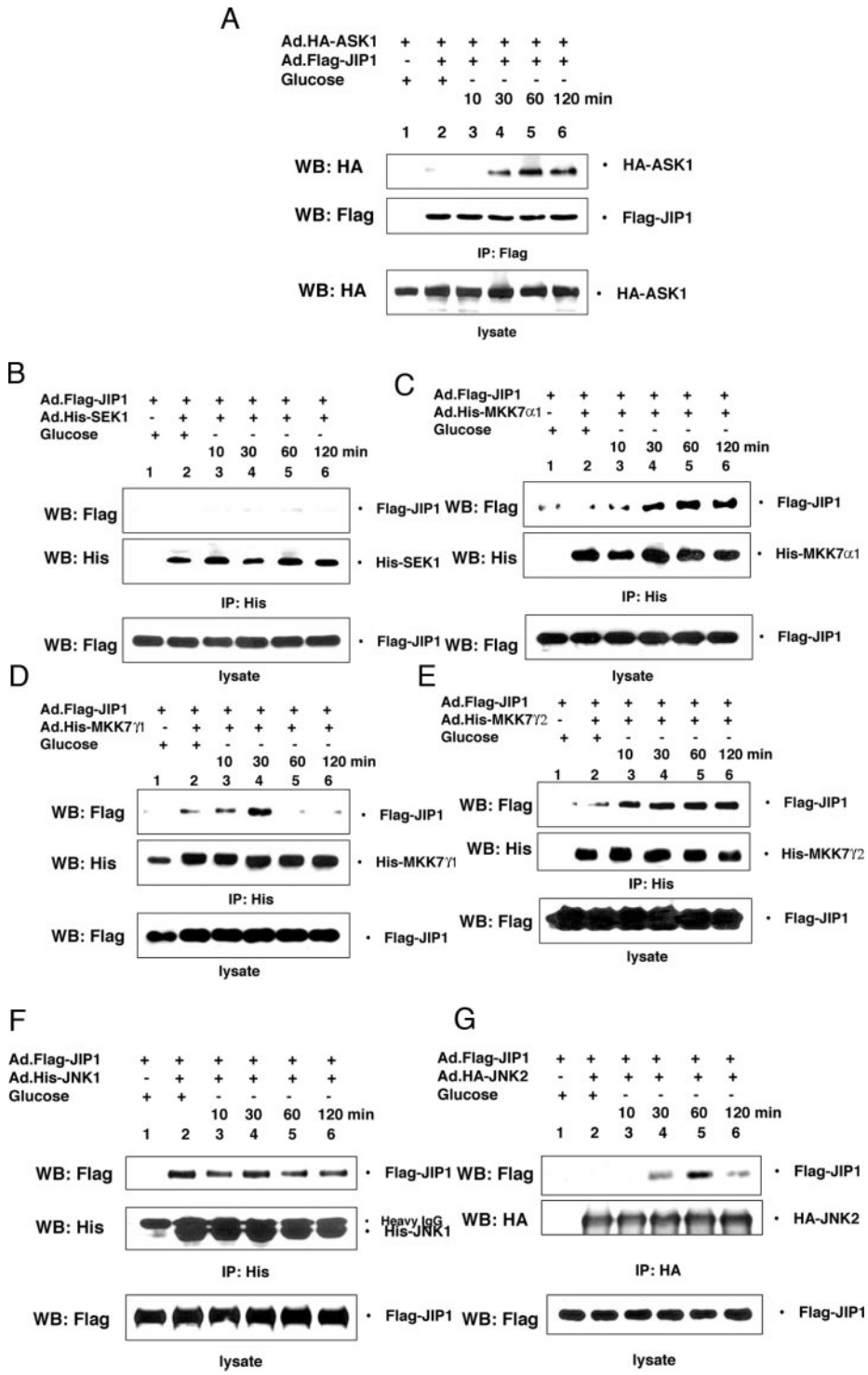

FIG. 3. **Interaction between JIP1 and various types of the ASK1-MEK-JNK signaling proteins during glucose deprivation.** DU-145 cells were co-infected with adenoviral vector containing FLAG-tagged JIP1 and HA-ASK1, His-MKK7α1, His-MKK7γ1, His-MKK7γ2, His-SEK1, HA-JNK1, or HA-JNK2 at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for various times (10–120 min). Cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody (*A*), anti-His antibody (*B–F*), or anti-HA antibody (*G*) and immunoblotted (*WB*) with anti-FLAG, anti-His, or anti-HA antibody (*upper panels*). The presence of HA-ASK1 or FLAG-JIP1 in the lysates was verified by immunoblotting (*lower panels*).

containing wild-type ASK1 (Fig. 5*A*) or mutant-type (kinase-inactive form) ASK1 (Fig. 5*B*) prior to glucose deprivation. Fig. 5 shows that glucose deprivation enhanced SEK1-JIP3 binding regardless of overexpressing either type of ASK1. SEK1 dissociated from JIP3 after 60 min of glucose deprivation in wild-type ASK1-overexpressed cells (Fig. 5*A*, *lane 5*). However, unlike overexpression of wild-type ASK1, overexpression of mutant type ASK1 prevented dissociation of SEK1 from JIP3 during prolonged glucose deprivation. We also observed that overexpression of wild-type ASK1 promoted JNK and SEK1 activation and sustained their activation during glucose deprivation (Fig. 5*A*). However, overexpression of mutant-type ASK1 suppressed JNK activation, and no detectable SEK1 phosphorylation on the Thr-261 residue was observed during glucose deprivation (Fig. 5*B*). We previously reported that glucose deprivation activates the ASK1-SEK1-JNK signal transduction pathway. Taken together, our results suggest that ASK1 kinase activity is not important for glucose deprivation-induced SEK1-JIP3 binding. Dissociation of activated (phosphorylated) SEK1 from JIP3 during prolonged glucose deprivation indicates that ASK1 kinase activity plays an important role in dissociation of SEK1 from JIP3.

Role of SEK1 in JNK Activation during Glucose Deprivation—To examine whether phosphorylation of SEK1 is essential for dissociation from JIP3, we employed site-directed

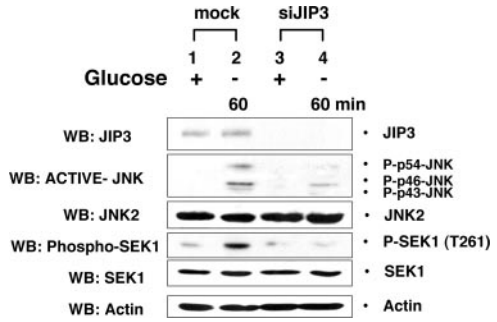

FIG. 4. **Role of JIP3 in the activation of SEK1 and JNK during glucose deprivation.** DU-145 cells were transfected with JIP3 siRNA or mock siRNA. After 36 h of incubation, cells were exposed to glucose-free medium for 60 min. Cell lysates were immunoblotted (*WB*) with anti-JIP3, anti-ACTIVE JNK, anti-JNK2, anti-phospho-SEK1, anti-SEK1, or anti-actin antibody. Actin was used to confirm that similar amounts of proteins were loaded in each lane.

mutagenesis to create a point mutation at residue Thr-259 (Thr → Ala) of mouse SEK1. Fig. 6 shows that wild-type SEK1 associated with JIP3 within 30 min of glucose deprivation and dissociated from JIP3 after 120 min of glucose deprivation. Unlike wild-type SEK1, mutant-type SEK1 did not dissociate from JIP3 during prolonged glucose deprivation. Interestingly, overexpression of mutant-type SEK1 suppressed glucose deprivation-induced JNK phosphorylation (Fig. 6). These results suggest that SEK1 has an effect on JNK activation.

Role of SEK1 in the Interaction between JNK2 and JIP1 during Glucose Deprivation—As shown in Figs. 2 and 3 and Table I, during prolonged glucose deprivation, SEK1 dissociates from JIP3, and JNK2 associates with JIP1. We postulated that activated SEK1, which dissociates from JIP3, regulates the interaction between JNK2 and JIP1. To test this hypothesis, we attempted to silence SEK1 expression by using siRNA of SEK1. DU-145 cells were stably transfected with either pSilencer control plasmid or pSilencer-siSEK1 vector. We selected several stable transfectants and chose one transfectant for further studies. Fig. 7 shows that the expression of SEK1 was effectively reduced in the siSEK#4 transfectant. To examine the role of SEK1 in the interaction between JIP1 and JNK2, pSilencer or siSEK1#4 transfectant was co-infected with Ad.FLAG-JIP1 and Ad.HA-JNK2. Fig. 7 shows that JNK2 bound to JIP1 in pSilencer transfectant but not in siSEK1#4 transfectant during glucose deprivation. Previous studies have shown that Thr-183 and Tyr-185 residues of JNK2 need to be phosphorylated for its activation (16). We postulated that phosphorylation of both residues is essential for JNK2-JIP1 interaction during glucose deprivation. To test this hypothesis, Thr-183 and Tyr-185 of JNK2 were replaced with alanine (T183A) and phenylalanine (Y185F), respectively. Fig. 8*A* shows that glucose deprivation increased the interaction between JIP1 and wild-type JNK2 or T183A mutant-type JNK2 but not Y185F mutant-type JNK2. These data suggest that phosphorylation of Tyr-185 residue but not that of Thr-183 is required for binding of JNK2 to JIP1 during glucose deprivation. Previous studies have demonstrated that activated JNK is involved in phosphorylation of JIP1 on the Thr-103 residue. To test this possibility, we employed site-directed mutagenesis to create a point mutation at residue Thr-103 (Thr → Ala) of JIP1. Fig. 8*B* shows that JIP1 was phosphorylated by only wild-type JNK2 but not by either T183A or Y185F mutant-type JNK2 during glucose deprivation. Data from immune complex kinase assays show that glucose deprivation-induced phosphorylation of JIP1 by JNK2 was reduced in T103A mutant-type JIP1 (Fig. 8*C*). These results suggest that phosphorylation of both

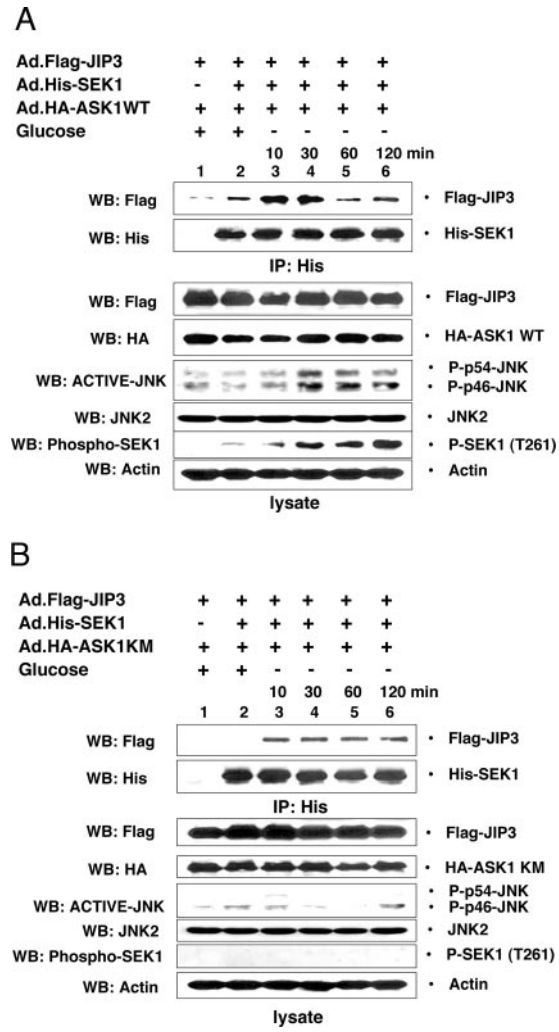

FIG. 5. **Role of ASK1 in the interaction between JIP3 and SEK1 during glucose deprivation.** DU-145 cells were co-infected with adenoviral vector containing His-tagged SEK1 (Ad.His-SEK1), FLAG-tagged JIP3 (Ad.FLAG-JIP3), and HA-tagged ASK1 wild type (Ad.HA-ASK1WT) (*A*) or HA-tagged ASK1 kinase-inactive form K709M (Ad.HA-ASK1KM) (*B*) at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for various times (10–120 min). Cell lysates were immunoprecipitated (*IP*) with anti-His antibody and immunoblotted (*WB*) with anti-FLAG or anti-His antibody (*upper panels*). The presence of FLAG-JIP3, HA-Akt1, phospho-JNK, JNK2, phospho-SEK1, or actin in the lysates was verified by immunoblotting (*lower panels*). Actin was used to confirm that similar amounts of proteins were loaded in each lane.

Thr-183 and Tyr-185 residue is necessary for full activation of JNK2 enzyme to phosphorylate its substrates such as JIP1 on Thr-103 residue.

Dissociation of Akt1 from JIP1 during Glucose Deprivation—Previous studies have shown that Akt1 associates with JIP1 (14). We hypothesized that JNK2-induced phosphorylation of JIP1 leads to dissociation of Akt1 from JIP1 during glucose deprivation. We also postulated that association of JIP1 with Akt1 inhibits the enzymatic activity of Akt1 and dissociation of Akt1 from JIP1 restores Akt1 enzyme activity. To test the hypothesis, interaction between Akt1 and wild-type JIP1 was examined during glucose deprivation. Fig. 9*A* shows that JIP1 associated with Akt1 in the presence of glucose. Prolonged glucose deprivation dissociated Akt1 from wild-type JIP1 (Fig. 9*A*) but not mutant-type JIP1 (T103A) (Fig. 9*B*). Fig. 9*C* shows that endogenous Akt1 catalytic activity, which was measured by using an Akt-specific substrate, Bad, was increased during prolonged (2–4-h) glucose

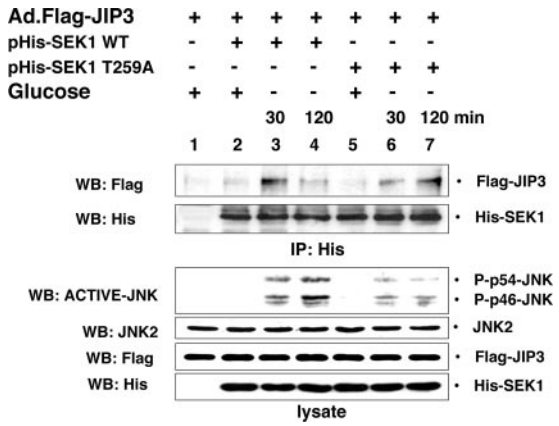

FIG. 6. **Interaction between JIP3 and wild-type SEK1 or mutant-type SEK1 (T259A) and JNK activation in wild-type SEK1 (pHis-SEK1-WT) plasmid or mutant-type SEK1 (pHis-SEK1-T259A) transfected DU-145 cells during glucose deprivation.** Cells were infected with Ad.FLAG-JIP3 at an MOI of 10 and transiently transfected with pHis-SEK1-WT (wild type) or pHis-SEK1-T259A (mutant type). After 48 h of incubation, cells were exposed to glucose-free medium for various times (30–120 min). Cell lysates were immunoprecipitated with anti-His antibody and immunoblotted (*WB*) with anti-FLAG or anti-His antibody (*upper panels*). The presence of phospho-JNK, JNK2, FLAG-JIP3, or His-SEK1 in the lysates was verified by immunoblotting (*lower panels*).

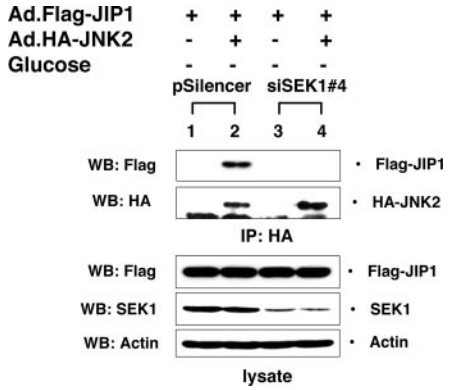

FIG. 7. **Role of SEK1 in interaction between JIP1 and JNK2 during glucose deprivation.** Control plasmid or pSilencer-siSEK1 stably transfected siSEK#4 cells were infected with Ad.FLAG-JIP1 and/or Ad.HA-JNK2 at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for 60 min. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted (*WB*) with anti-FLAG or anti-HA antibody (*upper panels*). FLAG-JIP1 in the lysates was verified by immunoblotting with anti-FLAG, anti-SEK1, or anti-actin antibody (*lower panels*).

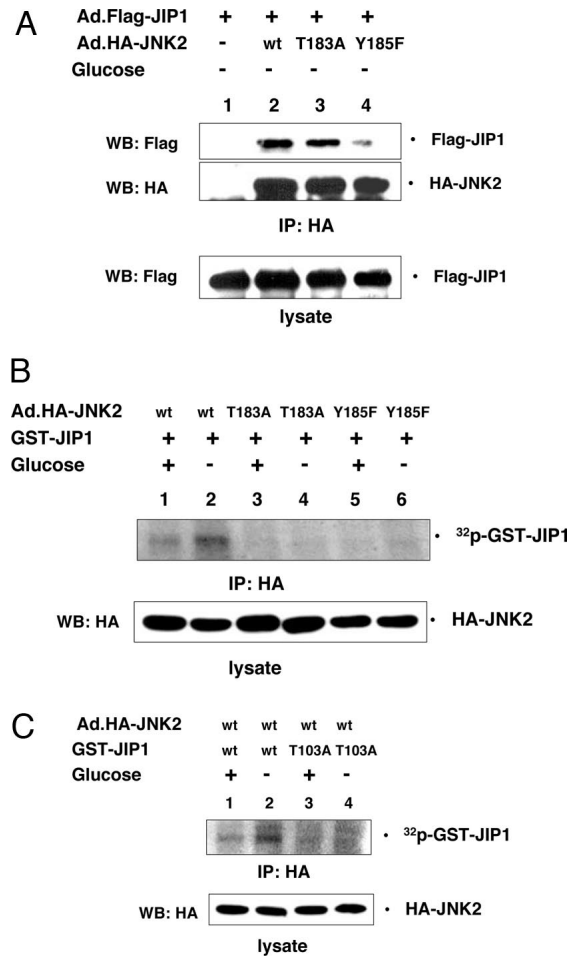

FIG. 8. **Role of Thr-183 and Tyr-185 residues of JNK2 in glucose deprivation-induced interaction between JNK2 and JIP1 (*A*) and JIP1 phosphorylation (*B* and *C*).** DU-145 cells were infected with Ad.HA-JNK2-wt, Ad.HA-JNK2-T183A, or Ad.HA-JNK2-Y185F at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for 1 h and lysed. *A*, cell lysates were immunoprecipitated with anti-HA antibody. Lysates were immunoprecipitated (*IP*) with anti-HA antibody and immunoblotted (*WB*) with anti-FLAG or anti-HA antibody (*upper panels*). The presence of FLAG-JIP1 in the lysates was verified by immunoblotting with anti-FLAG antibody (*lower panel*). *B*, to examine which types of JNK2 can phosphorylate JIP1, 0.5 μg of GST-JIP1 was incubated with immunoprecipitated HA-JNK2 in kinase buffer containing 100 μCi/ml [γ-^{32}P]ATP at 30 °C for 1 h. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography (*upper panel*). The presence of HA-JNK2 in the lysates was verified by immunoblotting with anti-HA antibody (*lower panel*). *C*, to investigate whether JNK2 phosphorylates JIP1 on Thr-103 residue, DU-145 cells were infected with Ad.HA-JNK2 at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for 1 h and lysed. Cell lysates were immunoprecipitated with anti-HA antibody, and then 0.5 μg of GST-JIP1 (wild type) or GST-JIP1-T103A (mutant type) was incubated with immunoprecipitated HA-JNK2 in kinase buffer containing 100 μCi/ml [γ-^{32}P]ATP at 30 °C for 1 h. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography (*upper panel*). The presence of HA-JNK2 in the lysates was verified by immunoblotting with anti-HA antibody (*lower panel*).

deprivation. The inhibitory role of JIP1 for Akt1 was further investigated by knockdown of JIP1 expression. DU-145 cells were stably transfected with either pSilencer control plasmid or pSilencer-siJIP1. Unlike pSilencer control plasmid-transfected cells, pSilencer-siJIP1 stably transfected siJIP1#3 cells contain a low level of JIP1 (Fig. 10*A*). Glucose deprivation-induced JNK activation was inhibited in siJIP1#3 cells (Fig. 10*B*). Akt1 activity as measured by the Akt-specific substrate, Bad, in siJIP1#3 cells was higher than that in pSilencer control plasmid-transfected cells. There was little or no change in the Akt1 activity in the presence or absence of glucose in siJIP1#3 cells (Fig. 10*C*).

Inhibition of SEK1/ASK1 by Akt1 during Glucose Deprivation—We further examined the role of Akt1 in the ASK1-SEK1-JNK signal transduction during glucose deprivation. We postulated that prolonged glucose deprivation-induced dissociation of Akt1 results in the suppression of the ASK1-SEK1-JNK signal transduction by inhibiting SEK1 and/or ASK1 activity. Figs. 11, *A* and *C*, shows that Akt1 associated with SEK1 and ASK1, respectively, during prolonged glucose deprivation (2 h but not 1 h). Fig. 11, *B* and *D*, shows that wild-type Akt1 phosphorylated SEK1 on Ser-80 residue and ASK1 on Ser-83 residue, respectively, during prolonged glucose deprivation. Loss of SEK1 and ASK1 activity was also observed during prolonged glucose deprivation as observed by the disappearance of active JNK (Fig. 11, *E* and *F*, *lane 4*). Overexpression of dominant negative Akt1 did not promote ASK1 phosphorylation (Fig. 11*D*) and inhibited the loss of

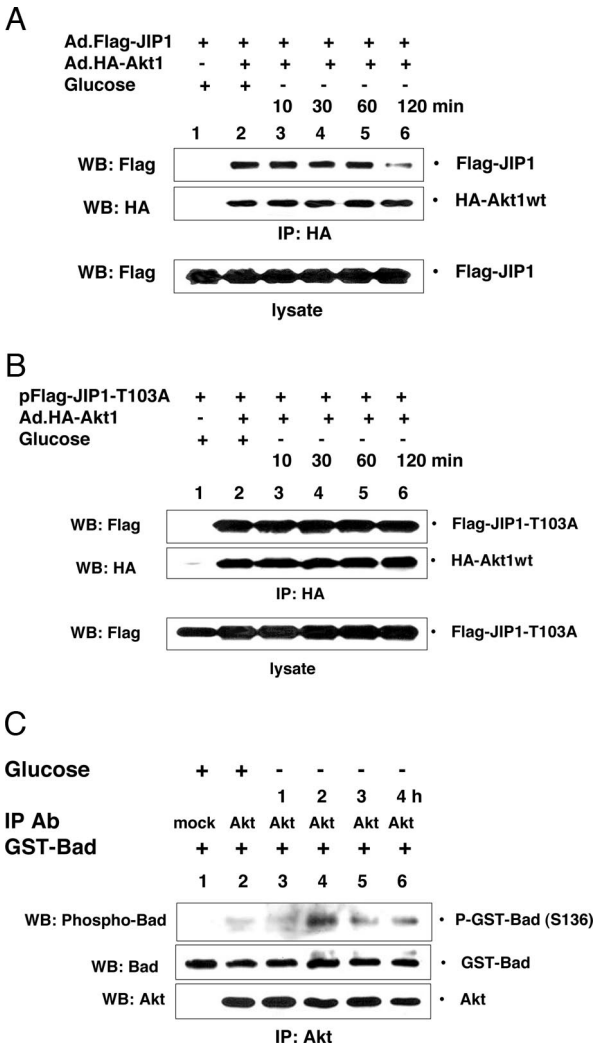

FIG. 9. **Interaction between Akt1 and JIP1 during glucose deprivation.** *A* and *B*, DU-145 cells were co-infected with adenoviral vector containing HA-tagged Akt1 (Ad.HA-Akt1) and FLAG-tagged wild-type JIP1 (Ad.FLAG-JIP1-WT) or mutant-type JIP1 (Ad.FLAG-JIP1-T103A) at an MOI of 10. After 48 h of infection, cells were exposed to glucose-free medium for various times (10–120 min). Cell lysates were immunoprecipitated (*IP*) with anti-HA antibody and immunoblotted (*WB*) with anti-FLAG or anti-HA antibody (*upper panels*). The presence of FLAG-JIP1 in the lysates was verified by immunoblotting (*lower panel*). *C*, DU-145 cells were exposed to glucose-free medium for various times (1–4 h). Lysates were immunoprecipitated with anti-Akt (mouse monoclonal antibody) antibody. Immunoprecipitates were analyzed for endogenous Akt1 catalytic activity *in vitro* using GST-Bad (Santa Cruz Biotechnology) protein as substrate. GST-Bad or phosphorylated GST-Bad was detected with anti-Bad or anti-phospho-Ser-136-Bad antibody, respectively. The presence of Akt1 was verified by immunoblotting with anti-Akt1 antibody.

SEK1 and ASK1 activity during prolonged glucose deprivation (Fig. 11, *E* and *F*, *lane 7*).

Model for the Role of SEK1-JNK2 and JIP1-Akt1 in ASK1-MEK-MAPK Signal Transduction during Glucose Deprivation—Fig. 12 shows a schematic diagram of a theoretical model based on the literature and data presented here. This model illustrates the sequential events that occur on scaffolding proteins during glucose deprivation: glucose deprivation → ASK1-JIP3 binding → phosphorylation of SEK1 at Thr-261 → phosphorylation of JNK2 at Tyr-185 → JNK2-JIP1 binding → MKK7 binding to JIP1 → phosphorylation JNK2 at Thr-183 → phosphorylation of JIP1 at Thr-103 → dissociation of Akt1 from JIP1 → phosphorylation of SEK1 at Ser-80 and ASK1 at Ser-83.

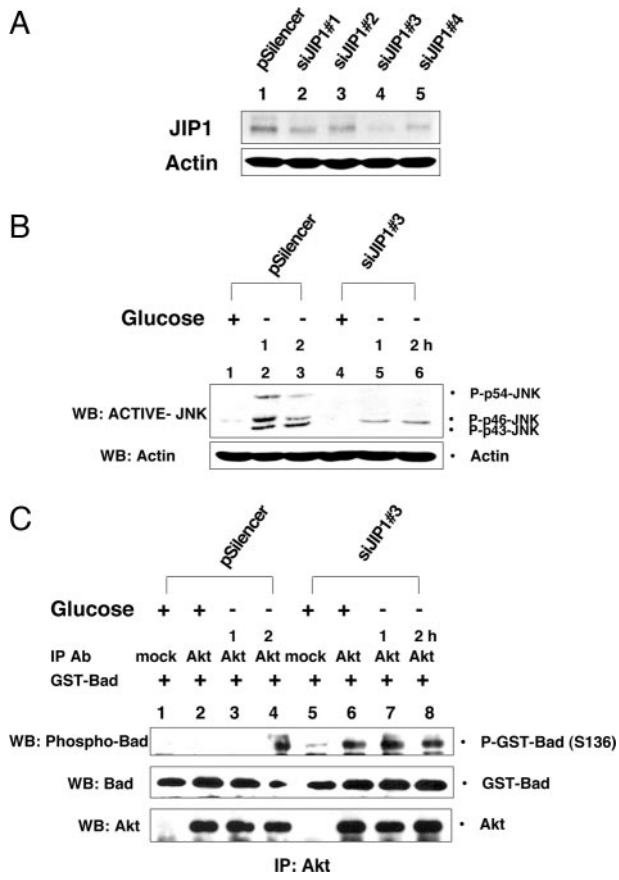

FIG. 10. **Glucose deprivation-induced JNK activation in control plasmid (pSilencer) or pSilencer-siJIP1 stably transfected DU-145 cells.** *A*, immunoblot of JIP1 expression in control vector transfected (pSilencer) or pSilencer-siJIP1 stably transfected (siJIP1#1, -2, -3, and -4) single cell clones from DU-145 cells. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-JIP1 antibody. *B*, control plasmid or pSilencer-siJIP1 stably transfected siJIP1#3 cells were exposed to glucose-free medium for 1 or 2 h. After incubation, cells were harvested, and Western blot analysis (*WB*) was performed with anti-ACTIVE JNK or anti-actin antibody as the loading control. *C*, control plasmid or pSilencer-siJIP1 stably transfected siJIP#3 cells were exposed to glucose-free medium for 1 or 2 h. After incubation, cells were harvested, and lysates were immunoprecipitated (*IP*) with anti-Akt (mouse monoclonal antibody) antibody. Immunoprecipitates were analyzed for Akt1 catalytic activity *in vitro* using GST-Bad (Santa Cruz Biotechnology) protein as substrate. GST-Bad or phosphorylated GST-Bad was detected with anti-Bad or anti-phospho-Ser-136-Bad antibody, respectively. The presence of Akt1 was verified by immunoblotting with anti-Akt1 antibody.

DISCUSSION

It is well known that biological regulatory systems usually have switchlike properties. Positive feedback loops and negative feedback loops may produce bistable systems under conditions of stress, as commonly exist in tumors. Our studies reveal that SEK1-JNK2 and Akt1 are involved in a cross-talk between JIP3 and JIP1 to negatively regulate the ASK1-SEK1-JNK signal transduction during prolonged glucose deprivation.

Previous studies have shown that JNK is activated by dual phosphorylation on the tripeptide motif Thr-*X*-Tyr (18). Two MAPKKs, SEK1 and MKK7, synergistically activate JNK (7, 8, 19, 20). SEK1 prefers the Tyr-185 residue, and MKK7 prefers the Thr-183 residue (17, 19). Our data demonstrate that full activation of JNK requires the phosphorylation of both residues (Fig. 8*B*). However, phosphorylation of JNK2 on Tyr-185 is prerequisite for the recruitment of JNK2 to JIP1 (Fig. 8*A*). Interestingly, knockdown of SEK1 mRNA and protein by siRNA for SEK1 leads to inhibition of JIP1-JNK2 binding dur-

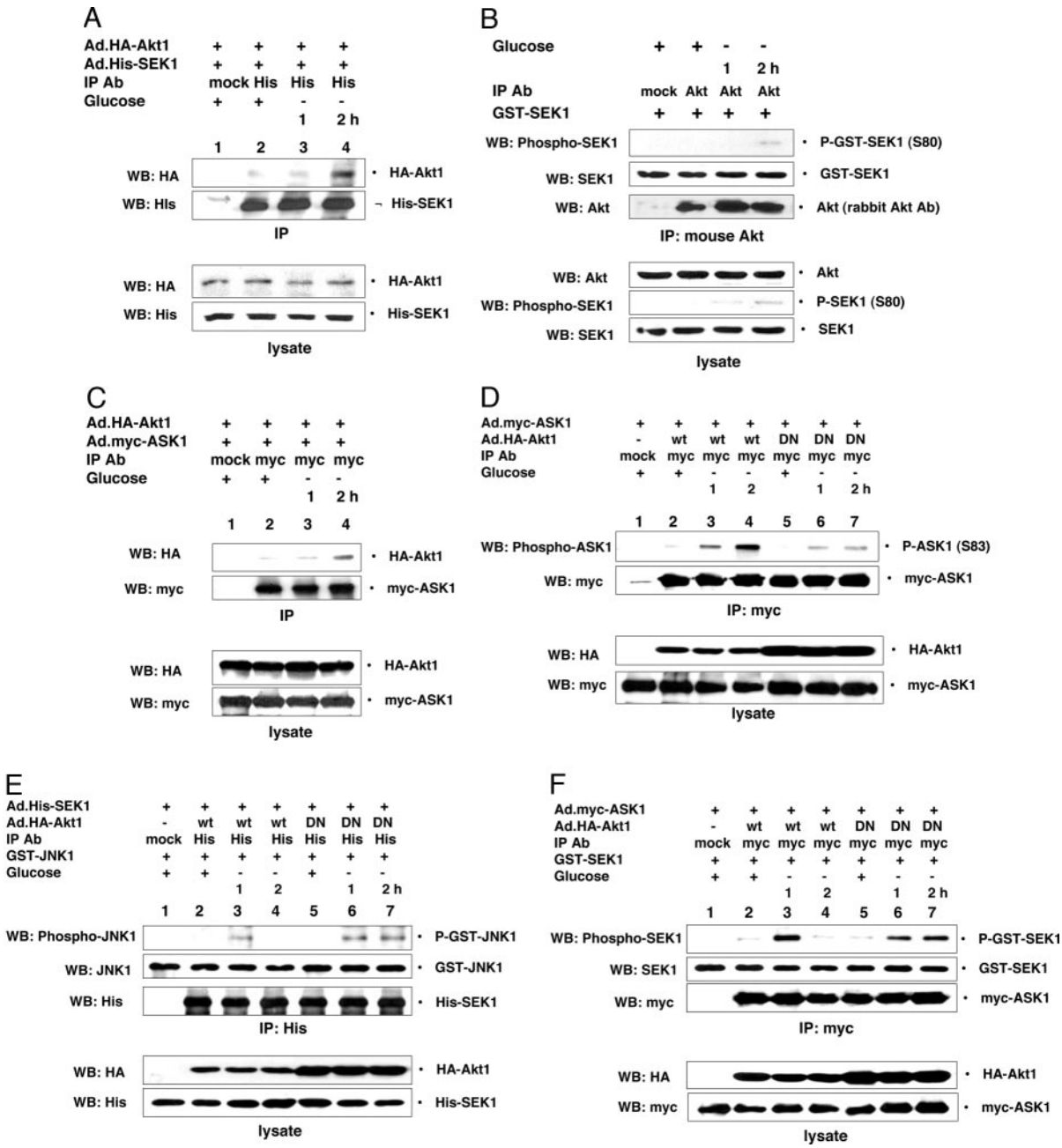

FIG. 11. **Interaction between Akt1 and SEK1 (or ASK1) and its role in SEK1/ASK1 enzyme activity during glucose deprivation.** *A* and *C–F*, DU-145 cells were co-infected with Ad.HA-Akt1 (wild type or dominant negative mutant type) and Ad.His-SEK1 (or Ad.Myc-ASK1) at an MOI of 10. After 48 h of incubation, cells were exposed to glucose-free medium for 1 or 2 h and lysed. Cell lysates were immunoprecipitated (*IP*) with anti-His (*A*) or anti-Myc antibody (*C*) and immunoblotted (*WB*) with anti-HA, anti-His, or Myc antibody (*upper panels*). The presence of HA-Akt1, His-SEK1, or Myc-ASK1 in the lysates were verified by immunoblotting (*lower panels*). *B* and *D*, DU-145 cells were exposed to glucose-free medium for 1 or 2 h and lysed. Endogenous Akt1 (*B*) or Myc-ASK1 (*D*) was immunoprecipitated with anti-Akt1 or anti-Myc antibody, respectively. Phosphorylation of SEK1 or that of ASK1 by Akt1 was detected with anti-phospho(Ser-80)-SEK1 or anti-phospho(Ser-83)-ASK1 antibody, respectively (*upper panels*). The presence of Akt1, phospho-SEK1, SEK1, HA-Akt1, or Myc-ASK1 was verified by immunoblotting (*lower panels*). *E* and *F*, after 48 h of incubation, cells were exposed to glucose-free medium for 1 or 2 h and then lysed. Lysates were immunoprecipitated with anti-His or anti-Myc antibody. To examine the catalytic activity of SEK1 or ASK1, 0.5 μg of GST-JNK1 or GST-SEK1 was incubated with immunoprecipitated His-SEK1 or Myc-ASK1, respectively, in kinase buffer containing ATP at 30 °C for 1 h. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-ACTIVE JNK or anti-phospho-Thr-261-SEK1 antibody. The presence of GST-JNK1, His-SEK1, GST-SEK1, or Myc-ASK1 in the immunoprecipitate was verified by immunoblotting (*upper panels*). The presence of HA-Akt1 (wild type, dominant negative), His-SEK1, or Myc-ASK1 in the lysates was verified by immunoblotting (*lower panels*).

ing glucose deprivation (Fig. 7). Taken together, these results suggest that SEK1-mediated JNK2 phosphorylation is necessary for the JIP1-JNK2 binding. Our observations were consistent with previous reports. It is well known that the JIP family of scaffold proteins associate with MAPK kinase kinase, MAPK kinase, and MAPK and create a functional signaling module to control the specificity of signal transduction (5). JIP1 scaffold facilitates JNK activation in a mixed lineage protein kinase-MKK7-dependent manner (9, 22–24). JIP-3 mediates signaling to JNK by interacting with ASK1, MEKK1, and MKK4 (7, 8). Indeed, our data show that glucose deprivation activates the JIP3-scaffolded ASK1-SEK1 signaling pathway and subsequently phosphorylates JNK2 on Tyr-185 but not Thr-183. Phosphorylated JNK2 on Tyr-185 binds to JIP1 and

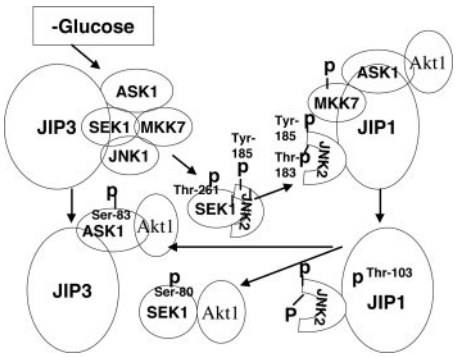

FIG. 12. **A schematic model for the involvement of JIP3, SEK1/MKK7, JNK1, JNK2, and JIP1 during glucose deprivation.**

then phosphorylation of JNK2 on Thr-183 probably occurs by the mixed lineage protein kinase-MKK7 signal. Although JIP3 and JIP1 are structurally distinct from each other, cross-talk underlies the relationship between the two scaffold proteins.

It is well known that Ser/Thr-directed protein kinases of the Akt family (Akt1 to -3) are important mediators of cell survival in response to growth factors including insulin and insulin-like growth factor I (25–28). Akt is activated by phosphoinositide-dependent kinase 1 and 2 through phosphorylation at Thr-308 and Ser-473 residues (29, 30). A number of proapoptotic proteins have been identified as direct Akt substrates, including Forkhead transcription factors, caspase-9, glycogen synthase kinase 3, and Bad (31–38). The proapoptotic function of these molecules is suppressed upon phosphorylation by Akt. Kim *et al.* (14) and Fig. 9*A* have revealed an interaction between Akt1 and JIP1. Data from our studies reveal that JIP1 negatively regulates Akt by means of protein-protein interactions (Figs. 9 and 10). Our observations somewhat contradicted a recent report (39) that JIP1 activates Akt1. At the present time, we can only speculate that this discrepancy is due to differences in analytical methods (immunblotting assay *versus* immune complex kinase assay). Immunoblotting with phosphospecific Akt antibodies may not truly reflect the catalytic activity of Akt. Data from immunoblot assays illustrate that phosphorylated (active form) Akt1 binds to JIP1; however, data from immune complex kinase assays demonstrate that the enzymatic activity of Akt1 is suppressed by binding with JIP1. The Akt1-JIP1 interaction is decreased concomitantly with an increase in an association between JIP1 and JNK (14). The present studies reveal that JNK2-dependent JIP1 phosphorylation on Thr-103 regulates JIP1-Akt1 binding affinity (Figs. 8 and 9). These results are consistent with previous reports, which demonstrate that JNK-mediated phosphorylation of JIP1 on Thr-103 is essential for the regulation of dual zipper-bearing kinase (DLK) association with JIP1 (11). Dissociation of DLK from JIP1 results in subsequent DLK oligomerization, autophosphorylation, and ultimately module activation of DLK (11). However, unlike DLK, phosphorylated Akt1 (active form) binds to JIP1, and dissociation of Akt1 from JIP1 results in the restoration of Akt1 enzyme activity (Fig. 9*C*). Previous studies reported that JNK activity can be antagonized by Akt kinase activity in numerous cellular systems (12–15). This is probably due to inhibition of ASK1 and/or SEK1 activity by Akt1. Our studies and literature have shown that Akt1 interacts with ASK1 or SEK1 and inhibits ASK1 or SEK1 enzyme activity by phosphorylating ASK1 on Ser-83 residue (40) (Fig 11*D*) or SEK1 on Ser-80 residue (41) (Fig. 11*B*), respectively. Taken together, Akt1-mediated inhibition of SEK1 and/or ASK1 may act as a negative regulatory feedback loop for the ASK1-MEK-MAPK signal transduction. Moreover, recent studies have demonstrated that Akt phosphorylates and activates ARK5, a member of the AMP-activated protein kinase family, during glucose deprivation (42, 43). Activated ARK5 phosphorylates ATM, a tumor suppressor, and subsequently leads to the activation of p53 by phosphorylation (42). The activation of ARK5, which is triggered by Akt during glucose deprivation, suppresses caspase activation and prevents cell death (21, 44). It is possible that Akt-activated ARK5 is involved in the negative regulatory feedback loop. This possibility needs to be investigated in the future.

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